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Antibiotics and Suppression of Lymphocyte Function in Vitro

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The effects on the mitogenic response of human T lymphocytes were studied for 20 different antibiotics. No apparent inhibitory effect could be detected for penicillins, cephalosporins, aminoglycosides, chloramphenicol, sulfamethoxazole, trimethoprim, nalidixic acid, and 5-fluorocytosine. There were effects at high concentrations with erythromycin, clindamycin, and rifampin, and these antibiotics could also be shown to depress the mitogenic response of B lymphocytes. With fusidic acid, nitrofurantoin, and doxycycline there was an inhibiting effect at low concentrations on the mitogenic responses of B and T lymphocytes and on in vitro antibody production. Protein synthesis in unstimulated lymphocytes was also inhibited. Some antibiotics thus may impair the function of human lymphocytes in vitro.

Antibiotics, such as actinomycin D and doxorubicin, are used as immunosuppressive agents, and some antibiotics used in the treatment of bacterial and fungal infections have also been shown to depress granulocyte and lymphocyte functions in vitro (7, 11-13, 15, 19, 20-22, 25-27) and the immune response in humans and experimental animals (2, 3, 8, 16, 21, 23).

The in vitro effects of a wide range of antibiotics on granulocyte chemotaxis and lymphocyte function were recently reported from our laboratory (11, 12). The aim of the present study was to extend the investigations on the in vitro functions of human B and T lymphocytes in the presence of commonly used antibiotics.

MATERIALS AND METHODS

Antibiotics. Fresh stock solutions (in RPMI 1640 medium) of the following preservative-free antibiotics were used: aminobenzylpenicillin, benzylpenicillin, and carbenicillin (Astra A. B., Södertälje Sweden; batches 175, 61062-01, and 50428-02); cefazolin and cephalothin (Lilly Research Centre Ltd., London, England; batches 7kk43 and 215-DJ 2-1); chloramphenicol (Carlo Erba s.p.a., Milan, Italy; batch 73 0767); clindamycin hyclate (The Upjohn Co., Kalamazoo, Mich.; batch U-21251 FIEK 55); duoxycycline hyclate (Pfizer Inc., New York, N.Y.; batch 312-58709); erythromycin (Upjohn; batch 2324 W); 5-fluorocytosine (Roche Diagnostics, Nutley, N.J.; batch 322-744); fusidic acid (Lövens läkemedel, Malmö, Sweden; batch 22194); gentamicin (San-bolagen A. B., Malmö, Sweden; batch 6694); kanamycin (AB Ferrosan, Malmö, Sweden; batch 03205 Z 9); lymecycline (Carlo Erba; batch 6 D 5154 9764); nalidixic acid (Winthrop Laboratories, New York, N.Y.; batch KL 1770); nitrofurantoin (Pharmacia A. B., Uppsala, Sweden; batch T 8533); rifampin (AB Ferrosan; batch 4422 T); sulfamethoxazole (AB Kabi, Stockholm, Sweden; batch 10708-52); tetracycline chloride (Dumex Ltd., Copenhagen, Denmark; batch 308 926); and trimethoprim (The Wellcome Foundation Ltd., London; batch 55 034).

Lymphocytes and culture media. Blood was drawn from healthy volunteers with heparin as an anticoagulant. After dilution with an equal volume of RPMI 1640 (Gibco Bio-Cult, Ltd., Edinburgh, Scotland), the blood was centrifuged on Ficoll-Isopaque (Lymphoprep; Nyegaard & Co. A/S, Oslo, Norway) at 400 × *g* for 16 min and the lymphocytes were harvested from the interface, washed twice in RPMI 1640, and finally suspended in serum-free RPMI 1640 supplemented with 2 mmol of L-glutamine per ml. Leucine-free RPMI 1640 was used in [^{14}C] leucine incorporation experiments.

Culture conditions. Triplicate samples were cultured either in plastic tubes (Falcon Plastics, Oxnard, Calif.; no. 2058) containing 5 × 10^5 lymphocytes in 1 ml of culture medium or in microtiter plates (Linbro Scientific Inc., Hamden, Conn.) containing 2.5 × 10^5 lymphocytes per culture in 200 μl of culture medium. Antibiotics were added at the beginning of the experiments, unless otherwise stated, to give final concentrations of 1, 10, 25, and 50 μg/ml. The lymphocytes were incubated usually for 3 days in 5% CO_2 at 37°C. With 50 μg of the different antibiotics per ml, the pH of the culture medium was unchanged after 3 days of incubation.

Mitogens. Phytohemagglutinin (PHA) (Wellcome, Ltd., London, England) was used in final concentrations of 1 and 0.5 μg/ml. The Cowan I strain of *Staphylococcus aureus* was used as a B-cell mitogen (14). An overnight culture of bacteria in CCY broth (1) was killed in 0.5% formaldehyde for 3 h at room temperature and heat treated at 80°C for 3 min. The killed bacteria were added to give a final concentration of 1/1,000 to 1/2,000 (50 to 100 bacteria per lymphocyte).

Measurement of deoxyribonucleic acid and protein syntheses. A 0.5- to 1-μCi amount of [^{3}H] thymidine (NET-027Z; specific activity, 48 Ci/mmol;

New England Nuclear Corp. [NEN], Boston, Mass.) or [^{14}C]leucine (NEC-279; specific activity, 270 mCi/mmol, NEN) in RPMI 1640 was added 24 h before the cultures were harvested onto glass fiber filters, using a Skatron harvesting machine (Skatron A/S, Lierbyen, Norway). The filters were dried and transferred to scintillation vials containing 4 ml of Insta-Fluor solution (Packard Instrument Co., Inc., Rockville, Md.). The radioactivity was measured in a Packard Tri-Carb liquid scintillation counter, and data were calculated from the median of triplicate cultures and expressed as percentages of the antibiotic-free controls.

Assay for plaque-forming cells. Plaque-forming cell assays were performed with a modification of the method described by Fauci and Pratt (10). Ten million lymphocytes in 20 ml of culture medium with 5% human AB serum (absorbed with sheep erythrocytes) were cultured with *S. aureus* Cowan I (final dilution, 1/1,000) in plastic bottles (Nunch Inc., Copenhagen, Denmark). Antibiotics were added in concentrations of 1, 10, and 25 μg/ml, and a control culture without antibiotics was always included. After incubation for 6 days in 5% CO_2 at 37°C the lymphocytes were washed and adjusted to 2×10^7 cells per ml in antibiotic-free RPMI 1640. To 200 μl of 0.5% agar (Difco Laboratories, Detroit, Mich.) in Hanks balanced salt solution containing 0.05% diethylaminoethyl-dextran (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) were added 25 μl of a suspension of sheep erythrocytes diluted 1:5 in Hanks balanced salt solution, 100 μl of lymphocyte suspension, and 25 μl of guinea pig complement previously absorbed with sheep erythrocytes and diluted 1:4 in phosphate-buffered saline. On a plastic petri dish was placed 100 μl of this mixture, and a glass cover slip (22 by 32 mm) was placed over it to give a thin layer of agar. The plates were incubated for 3 h at 37°C. Plaque-forming cells were counted, using a stereomicroscope (Wild M5; Wild-Heerbrug Inc., Heerbrug, Switzerland). Representative plaques were checked for a central lymphocyte. Triplicate plaque assays were performed, and the results were expressed as percent plaque-forming cells in the presence of different antibiotic concentrations compared with the percent in control cultures without antibiotics.

Statistical methods. For statistical evaluation we used the Student's *t* test for comparison of a series of measurements with an expectation.

RESULTS

Effects on the mitogenic response of T lymphocytes. The effects of 20 different antibiotics on [^{3}H]thymidine incorporation in PHA-stimulated human lymphocytes were studied. Fourteen antibiotics (aminobenzylpenicillin, benzylpenicillin, carbenicillin, cefazolin, cephalothin, chloramphenicol, 5-fluorocytosine, gentamicin, kanamycin, lymecycline, nalidixic acid, sulfamethoxazole, tetracycline chloride, and trimethoprim) did not inhibit the PHA response of lymphocytes when they were cultured in the presence of 50 μg of each antibiotic per ml for 3 days. With these antibiotics [^{3}H]thymidine incorporation in lymphocytes differed less than 20% compared with that in control lymphocytes cultured in the absence of antibiotics, and this was considered indicative of no significant inhibitory or stimulatory effect. Antibiotics giving more than 20% depression of PHA response of lymphocytes were retested in concentrations from 1 to 50 μg/ml. Lymphocytes from 6 to 12 different healthy blood donors were used, and the results are presented in Table 1. The numbers of viable lymphocytes after 3 days of incubation with 50 μg of the respective antibiotics per ml were not influenced. For clindamycin and erythromycin dose-dependent inhibitions of lymphocyte response to PHA were detected (Table 1). At a concentration of 50 μg/ml the responses were reduced to about 50% of the control. With fusidic acid there was an inhibition with a concentration of 1 μg/ml, and the depression was highly significant at a concentration of 10 μg/ml, which depressed the response to less than 50% of the control. Nitrofurantoin at a concentration of 10 μg/ml or rifampin at a concentration of 50 μg/ml significantly depressed the mitogenic response, which was reduced to about 30 or 20%, respectively. Doxycycline had a highly significant effect at a concentration of 10 μg/ml. Four different tetracyclines were compared in the same experiments, and one representative experiment is shown in Fig. 1. There

TABLE 1. *[^{3}H]thymidine incorporation in PHA-stimulated lymphocytes in the presence of different antibiotics.*

Antibiotic	% of control[a] at antibiotic final concn of:			
	1 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml
Erythromycin	98.3 ± 4.2	87.1 ± 8.4	65.2 ± 9.2[b]	48.3 ± 9.3[c]
Clindamycin	97.2 ± 5.4	83.2 ± 7.8	76.2 ± 10.0	57.4 ± 11.6[b]
Fusidic acid	80.5 ± 3.7[c]	43.8 ± 7.4[d]	14.5 ± 3.6[d]	2.5 ± 0.8[d]
Nitrofurantoin	102.2 ± 5.8	29.5 ± 10.3[d]	10.3 ± 7.7[d]	8.5 ± 7.0[d]
Rifampin	95.8 ± 7.9	84.0 ± 10.5	58.8 ± 12.7[b]	19.7 ± 4.6[d]
Doxycycline	99.0 ± 6.1	46.8 ± 10.6[d]	9.2 ± 7.0[d]	1.1 ± 0.3[d]

[a] Percentages (± standard error of the mean) of control value obtained in the absence of antibiotics.
[b] $0.01 < P < 0.05$.
[c] $0.001 < P < 0.01$.
[d] $P < 0.001$.

was no effect of tetracycline chloride or lymecycline at a concentration of 25 μg/ml, whereas there was a pronounced effect of minocycline or doxycycline.

Effects on the mitogenic response of B lymphocytes. The Cowan I strain of *S. aureus* was used as a B-lymphocyte mitogen, and the inhibiting effect was studied for antibiotics that had been shown to inhibit the mitogenic response of T lymphocytes. Aminobenzylpenicillin and carbenicillin were included as controls in these experiments, and there was no effect of these antibiotics, whereas erythromycin, clindamycin, fusidic acid, nitrofurantoin, rifampin, and doxycycline all inhibited the B-lymphocyte mitogenic response. The degrees of suppression with different concentrations of antibiotics were almost identical to those of T lymphocytes.

Kinetics of PHA response of lymphocytes. Figure 2 demonstrates the effects on the PHA responses of lymphocytes cultured in the presence of fusidic acid, nitrofurantoin, rifampin, and doxycycline for 1 to 4 days at concentrations of 10 μg/ml. Thymidine always was added at day 4 and the pulsing time was 24 h. There was a strong effect when the antibiotics were present from the day 1 or 2, a moderate effect when the antibiotics were present from the day 3, and no effect when they were added at day 4.

Reversibility of lymphocyte mitogenic response. When lymphocytes were precultured in the presence of fusidic acid, nitrofurantoin, rifampin, and doxycycline for 2 days at a concentration of 50 μg/ml, the responses to PHA and the Cowan I strain of *S. aureus* subsequently

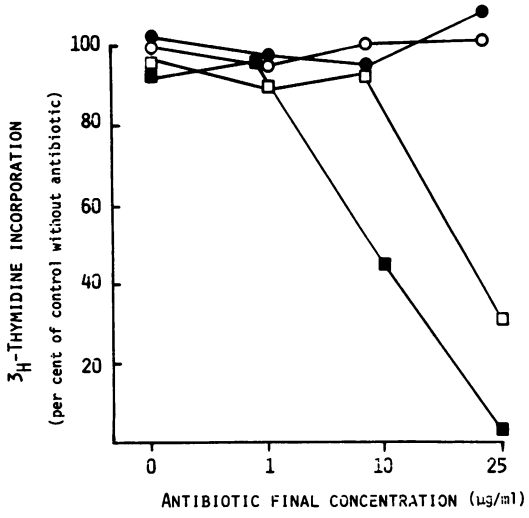

FIG. 1. *Effects on lymphocyte response to PHA by tetracycline chloride* (○), *lymecycline* (●), *minocycline* (□), *and doxycycline* (■).

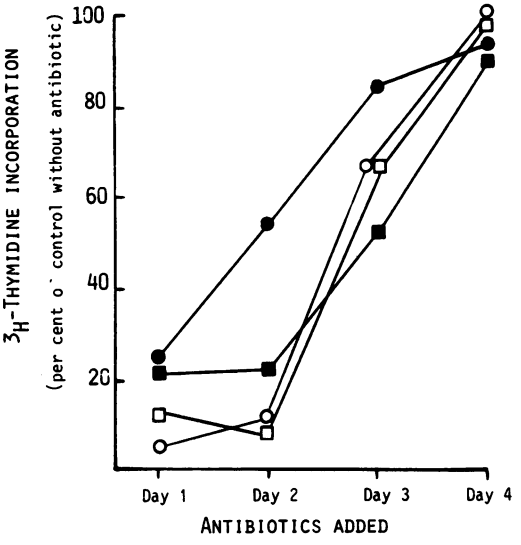

FIG. 2. *Effect on lymphocyte response to PHA after incubation with 10 μg/ml for 1 to 4 days with nitrofurantoin* (○), *rifampin* (●), *doxycycline* (□), or fusidic acid (■).

added were strongly depressed. The reversibility of the depressed lymphocyte function was studied by washing the lymphocytes after 2 days of incubation with the above-mentioned antibiotics and aminobenzylpenicillin as a control and adjusting them to the same number of viable cells per milliliter. The lymphocytes were then stimulated by PHA and the Cowan I strain of *S. aureus* (Fig. 3). With fusidic acid the lymphocyte response was the same as that of control lymphocytes cultured with aminobenzylpenicillin or without antibiotics. Rifampin did not interfere with the response to PHA, whereas the response to the Cowan I strain of *S. aureus* was increased in all four experiments performed (140 to 280% of the control). The responses of cells that had been preincubated with doxycycline and nitrofurantoin and then washed were profoundly depressed.

Effects on protein synthesis in unstimulated lymphocytes. Protein synthesis in unstimulated lymphocytes was studied by measuring [^{14}C]leucine incorporation in lymphocytes cultured for 3 days in the presence of fusidic acid, nitrofurantoin, rifampin, doxycycline, and aminobenzylpenicillin as a control. Figure 4 demonstrates a representative experiment out of four. With doxycycline or nitrofurantoin at a concentration of 25 μg/ml, the protein synthesis was only about 20% of the control, and with either of these two antibiotics protein synthesis was also depressed by 10 μg/ml. The incorporation of [^{14}C]leucine in the presence of 25 μg of

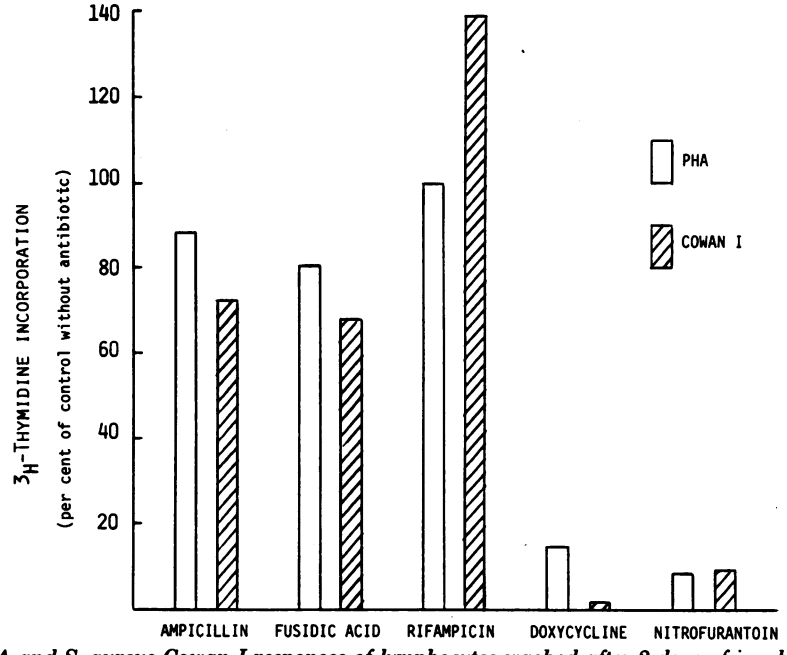

FIG. 3. *PHA and S. aureus Cowan I responses of lymphocytes washed after 2 days of incubation with 50 μg of different antibiotics per ml.*

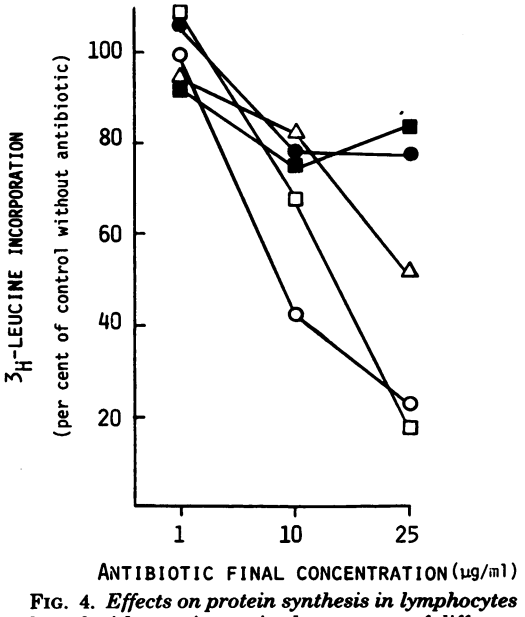

FIG. 4. *Effects on protein synthesis in lymphocytes cultured without mitogen in the presence of different concentrations of nitrofurantoin* (○), *rifampin* (●), *doxycycline* (□), *ampicillin* (■), *and fusidic acid* (△).

fusidic acid per ml was 50% of the control. Rifampin had no apparent inhibitory effect. The kinetics of the inhibition of protein synthesis was studied by incubation of lymphocytes with 25 μg of fusidic acid per ml added at days 1, 2, and 3. When added on day 1 the incorporation of [^{14}C]leucine was decreased to 50%; when added on days 2 and 3 there was no effect.

Effects on in vitro antibody production. With the use of the Cowan I strain of *S. aureus* as a polyclonal B-lymphocyte activator and a plaque assay for detection of antibody-producing cells, the effects on antibody production by fusidic acid, nitrofurantoin, and doxycycline were studied. Rifampin was not included in these experiments because of its poor stability at 37°C after 6 days. In these experiments the lymphocytes were incubated with 1, 10, and 25 μg of the respective antibiotic per ml for 6 days. In Fig. 5 are shown representative results of 4 to 6 experiments. There was a complete depression of antibody production for doxycycline or nitrofurantoin at a concentration of 10 μg/ml or for fusidic acid at 25 μg/ml. Aminobenzylpenicillin and gentamicin were included as controls in these experiments, and with these antibiotics there was no effect on antibody production.

DISCUSSION

Apart from our recent report (11), no study seems to exist where the influences of a wide range of commonly used antibiotics on the in vitro function of lymphocytes have been compared. In this extended investigation it was found that 14 out of 20 antibiotics tested did not impair the in vitro mitogenic response of human

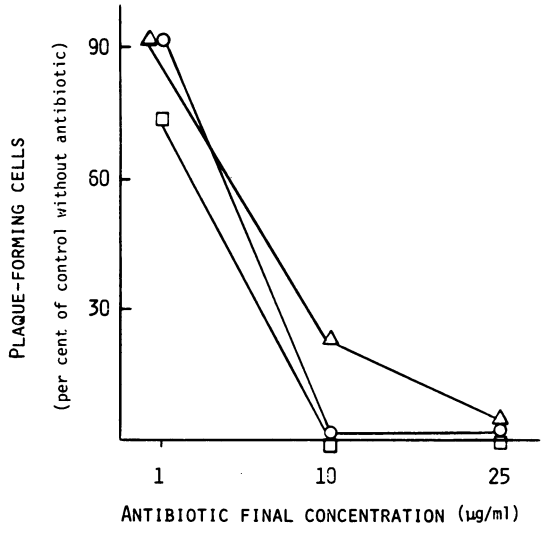

FIG. 5. *Antibody production after 6 days of lymphocyte incubation with different concentrations of nitrofurantoin* (○), *doxycycline* (□), *and fusidic acid* (△).

T lymphocytes even in concentrations exceeding those achieved in serum during treatment. We could not show any immunosuppressive effects by chloramphenicol, for which data are conflicting (9, 22). No immunosuppression was found with trimethoprim alone. This antibiotic has been shown to be immunosuppressive in combination with sulfamethoxazole (2, 15). Among the different cephalosporins we only tested cephalothin and cefazolin, for which no immunosuppressive effect was shown, which is in accordance with the report by Chaperon and Sanders (5). We have not found convincing data in the literature showing good intracellular penetration for any of these 14 noninhibiting antibiotics. For some of these antibiotics a target for the antibiotic within the lymphocyte may exist, and the noninterference thus might depend on poor penetration.

Erythromycin and clindamycin only inhibited lymphocyte function at high concentrations. Erythromycin and the clindamycin analog lincomycin have been shown to penetrate well into HeLa cells (4); however, no information is available on the penetration of macrolide antibiotics into human leukocytes. The immunosuppressive effects of these antibiotics are probably not of clinical relevance in immunocompetent individuals. They both act in bacteria by inhibition of protein synthesis, and it is of theoretical interest that they might act in a similar way in human cells.

With doxycycline there was a highly significant depression of the mitogenic responses of both B and T lymphocytes with a concentration of 10 μg/ml, and a similar effect was seen with minocycline, whereas the older, less lipid-soluble tetracycline chloride and lymecycline in concentrations up to 25 μg/ml did not interfere with the mitogenic response of lymphocytes. Our findings are in good agreement with those of Thong and Ferrante (25), who showed a strong lymphocyte depression by doxycycline at 10 μg/ml but none by tetracycline and oxytetracycline. The effect of doxycycline was not reversible, as the lymphocytes could not be stimulated when washed after 2 days of incubation with 50 μg/ml. This irreversible effect was not due to cell death, as the viability of the lymphocytes was not affected. Tetracyclines have been shown to penetrate well into human leukocytes, giving intracellular accumulation (4). In contrast to some other antibiotics, such as fusidic acid, tetracyclines are not released from HeLa cells when they are incubated in the absence of antibiotics.

Antibody production by lymphocytes incubated for 6 days with doxycycline at a concentration of 10 μg/ml was completely depressed. Tetracyclines act by inhibition of protein synthesis in bacteria, and we could show inhibition of protein synthesis in unstimulated lymphocytes by doxycycline. The effect by doxycycline on lymphocyte function was delayed, as doxycycline had to be present for 2 days or more in order to suppress the mitogenic response. This in vitro finding is in good agreement with a delay of onset of the catabolic effect in patients shown by Shils (24) and with the clinical observation by Korkeila (17) that normal doses of tetracycline could completely abolish the effect of parenteral nutrition by inhibiting the utilization of amino acids for protein synthesis. Doxycycline also affects tissue metabolism (18), but this effect is less likely to be manifested with doxycycline, as this drug does not accumulate. We have earlier shown that doxycycline inhibits the chemotactic response of human leukocytes in vitro (12), and the combined action on protein metabolism, and lymphocyte and leukocyte functions may possibly be potentially dangerous to, for example, postoperative patients, who require an effective anabolism and a good immunological defense.

Rifampin significantly depressed the mitogenic responses of B and T lymphocytes at a concentration of 50 μg/ml, and this is in good accordance with earlier reports (3, 7, 8, 16, 20, 21, 23). As for the other antibiotics, the effects were not immediate. When lymphocytes were washed after 2 days of incubation with 50 μg of rifampin per ml and then stimulated, the T-lymphocyte response was unaffected, whereas

the B-lymphocyte response was increased. This increase in B-lymphocyte response so far is unexplained. The suppression of the mitogenic response and of protein synthesis in unstimulated lymphocytes was less pronounced with rifampin than with the other immunosuppressive antibiotics studied. Rifampin is degraded rather rapidly in solution at 37°C, and the effects of rifampin would probably have been more pronounced if the concentration of active drug was kept constant throughout each experiment.

Rifampin had an effect at concentrations sometimes achieved therapeutically. Only one preliminary report so far has been presented where the immunosuppressive effect of rifampin was evaluated in human beings (3). In this study it was found that the antibody responses to cholera vaccine were not significantly affected in a small number of volunteers given clinically recommended doses of rifampin. However, in experimental animals rifampin has been shown to depress the antibody response (3, 21). It has also been shown to prolong the survival of skin allografts in rabbits (23) and to inhibit the delayed hypersensitivity reaction in guinea pigs and rats (8, 16). We have shown a strong depression by very low concentrations of rifampin on the in vitro chemotactic response of human leukocytes (12). A combined effect on both leukocyte chemotaxis and lymphocyte function may thus explain the in vivo findings.

Nitrofurantoin at a concentration of 10 μg/ml profoundly affected the B- and T-lymphocyte responses to mitogens, antibody production, and protein synthesis in unstimulated lymphocytes. As with doxycycline, the effect on the mitogenic response could not be eliminated by washing the lymphocytes. The serum level during treatment is very low, so the clinical relevance of our in vitro findings probably is of little importance. The penetration of nitrofurantoin into human leukocytes has not been investigated. However, our results gave indirect evidence of intracellular penetration, and there may be a local effect on the lymph nodes draining the kidneys, as the concentration in renal lymph is much higher than that in serum (6).

Fusidic acid significantly depressed the mitogenic response of B and T lymphocytes at a concentration of 1 μg/ml. This effect was reversible, which is in agreement with the observation that fusidic acid penetrates well into HeLa cells and is rapidly and to a great extent released from the cells in antibiotic-free medium (4). Fusidic acid has also been shown to penetrate into human leukocytes (4). As with tetracyclines, rifampin, and nitrofurantoin, there was a delayed effect on the mitogenic response. With 25 μg of fusidic acid per ml the protein synthesis in unstimulated lymphocytes was depressed to about 50% of that in control lymphocytes cultured in the absence of antibiotics. The in vitro antibody production was also completely depressed when lymphocytes were cultured for 6 days in the presence of 25 μg/ml. A catabolic effect has been shown for fusidic acid, and interference with protein synthesis in human tissue cells has been suggested (28). Fusidic acid has been shown to depress the chemotactic response of human leukocytes in vitro (12), and the in vivo effects of the combined action on leukocyte and lymphocyte function should be further evaluated in animal experiments and clinical studies. The in vivo relevance of our in vitro findings is difficult to evaluate. However, experiments in progress in our laboratory with fusidic acid and rifampin have shown suppression of both cellular and humoral immunity in experimental animals.

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